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Differential inflammatory networks distinguish responses to bone marrow-derived versus adipose-derived mesenchymal stem cell therapies in vascularized composite allotransplantation

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BACKGROUND: Vascularized composite allotransplantation (VCA) is aimed at enabling injured individuals to return to their previous lifestyles. Unfortunately, VCA induces an immune/inflammatory response, which mandates lifelong, systemic immunosuppression, with attendant detrimental effects. Mesenchymal stem cells (MSC)—both adipose-derived (AD-MSC) and bone marrow–derived (BM-MSC)—can reprogram inflammation and have been suggested as an alternative to immunosuppression, but their mechanism of action is as yet not fully elucidated. We sought to gain insights into these mechanisms using a systems biology approach.

METHODS: PKH26 (red) dye-labeled AD-MSC or BM-MSC were administered intravenously to Lewis rat recipients of mismatched Brown-Norway hindlimb transplants. Short course tacrolimus (FK-506) monotherapy was withdrawn at postoperative day 21. Sera were collected at 4 weeks, 6 weeks, and 18 weeks; assayed for 29 inflammatory/immune mediators; and the resultant data were analyzed using Dynamic Network Analysis (DyNA), Dynamic Bayesian Network (DyBN) inference, and Principal Component Analysis.

RESULTS: DyNA network complexity decreased with time in AD-MSC rats, but increased in BM-MSC rats. DyBN and Principal Component Analysis suggested mostly different central nodes and principal characteristics, respectively, in AD-MSC versus BM-MSC rats.

CONCLUSION: AD-MSC and BM-MSC are associated with both overlapping and distinct dynamic networks and principal characteristics of inflammatory/immune mediators in VCA grafts with short-course tacrolimus induction therapy. The decreasing inflammatory complexity of dynamic networks in the presence of AD-MSC supports the previously suggested role for T regulatory cells induced by AD-MSC. The finding of some overlapping and some distinct central nodes and principal characteristics suggests the role of key mediators in the response to VCA in general, as well as potentially differential roles for other mediators ascribed to the actions of the different MSC populations. Thus, combined in vivo/in silico strategies may yield novel means of optimizing MSC therapy for VCA. (*J Trauma Acute Care Surg.* 2017;83: S50–S58. Copyright © 2017 Wolters Kluwer Health, Inc. All rights reserved.)

KEY WORDS: Vascularized composite allotransplantation; mesenchymal stem cells; inflammation; systems biology; dynamic networks.

Vascularized composite allotransplantation (VCA) is an emerging therapy for devastating trauma in both combat and civilian settings, in selected subjects who have exhausted conventional reconstructive or alternate treatment options. Over 200 VCAs, including 115 hand and 35 face transplants, have been performed around the world in the past decade, with favorable immunologic, graft survival, and quality of life outcomes in most patients.[1] However, routine applicability and widespread impact of VCA for catastrophic combat or civilian trauma will only be realized if immunosuppressive risk is minimized and functional outcomes are maximized.

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Compelling experimental and clinical evidence in solid organ transplant and VCA studies supports the role of bone marrow–derived mesenchymal stem cells (BM-MSC) and adipose-derived mesenchymal stem cells (AD-MSC) in promoting modulation of the alloimmune response while augmenting nerve regeneration and tissue repair.[2–4] Such therapies have the potential to refine current immunosuppressive approaches, to optimize functional outcomes and to increase clinical applicability of VCA, thereby improving overall quality of life for injured service members or civilians with devastating and disabling injuries not amenable to conventional salvage/reconstructive strategies.

Inflammation is a key denominator in both trauma as well as transplantation. Tissue damage and ischemia-reperfusion injury that may occur in these two settings can induce a complex host response driven by various cell types that produce endogenous danger signals, such as alarmins or damage-associated molecular patterns, chemokines, and cytokines that disrupt the immune homeostasis between inflammatory and anti-inflammatory immune responses. Although, in transplantation, this manifests as the host versus graft response (rejection) or graft versus host response, in trauma such loss of immune homeostasis is manifested by a continuum of phenomena that involve overly exuberant systemic inflammation and attendant immune anergy. This dysregulated response can cause loss of immune competence in trauma patients, increasing the risk of nosocomial infection and sepsis in much the same way that

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immunosuppression affects transplant recipients to increase the risk of opportunistic infections.

The cellular and molecular mediators of the innate and adaptive immune system do not function in isolation but rather in complex and dynamic feedback interactions to either stimulate or suppress inflammation.[5] Great progress has been made in the understanding of how traumatic injury or transplantation influences the immune system and how inflammation and immune dysregulation affect in outcomes in either scenario, such as sepsis or rejection. T regulatory (Treg) cells are versatile and efficient regulators of the injury response and inflammation in both transplant[6] and trauma[5] settings. In a similar vein, cell therapies, such as AD-MSC or BM-MSC, are known to exert their immunomodulatory or tolerogenic effects through Treg cell–mediated mechanisms, especially of the adaptive immune response.[7,8]

The skin of VCAs, such as hand or face transplants, is a major locus for cellular inflammatory and immune reactivity mediated and propagated by cytokines and chemokines. Inflammation is thus a key driver of rejection post-VCA.[9] Though properly regulated inflammation allows for timely recognition and repair of traumatic injury and subsequent VCA, insufficient[10] or self-sustaining[11] inflammation can lead to long-lasting immune dysregulation.[12] This prolonged immune dysregulation and ongoing tissue damage, can, in turn, predispose to transplant rejection in various contexts,[13,14] including VCA.[9,15]

Taken together, insights into the intricate and complex dynamics underlying cellular, molecular, and genomic processes underlying acute rejection (AR) are critical to the success of immunomodulatory or tolerance strategies in VCAs. In prior studies, we demonstrated that dosing and timing of BM-MSC and AD-MSC cell therapy are key variables in influencing immunomodulatory and graft survival outcomes after VCA.[4] In the current study, our goal was to investigate whether these immunomodulatory effects as manifested by suppression of AR and prolongation of VCA survival are associated with specific effects on dynamic inflammatory network patterns after VCA. Using a systems biology approach, we examined dynamic networks and principal drivers of AR in VCA as a key to establishing foundational understanding of downstream chronic pathological processes including chronic rejection.

MATERIALS AND METHODS

Animals

All procedures involving animals complied with the regulations regarding the care and use of experimental animals published by the National Institutes of Health, and were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh.

Orthotopic Limb Transplantation Surgical Technique and Procedural Detail

Lewis rats received orthotopic hindlimb transplants from full Major Histocompatibility Complex-mismatched Brown-Norway (BN) donor rats. Isoflurane was used for inhalation anesthesia. Bilateral shaved hind limbs of BN donors were dissected through a circumferential skin incision, after ligation of epigastric vessels, dissection of femoral vessels, and transection at the level of the inguinal ligament. The two limbs were amputated at midfemoral level and served as donor limbs for two LEW recipients. Osteosynthesis was performed using an intramedullary 18-gauge needle. Sciatic nerves were coapted using 9-0 nylon sutures (Microsurgery Instruments, Inc., Bellaire, TX), and the muscles sutured with interrupted 4-0 Vicryl stitches (Ethicon Inc., Somerville, NJ). Microsurgical anastomosis of the femoral artery was performed with interrupted 11-0 nylon stitches, whereas a polyamide tube (RiverTech Medical, Chattanooga, TN) was used as cuff for the femoral vein, as reported earlier.

Isolation of BM-MSC and AD-MSC

The BM-MSCs were obtained from 6-week- to 8-week-old BN rats by flushing long bones with Roswell Park Memorial Institute 1640 medium (Lonza, Walkerville, MD). The cellular pellet was erythrocyte-depleted, centrifuged, resuspended in complete medium (Roswell Park Memorial Institute 1640 medium, 10% fetal bovine serum, 2.5 μM HEPES [Sigma-Aldrich], 1% penicillin-streptomycin, 1.25 mg/L amphotericin B, 1% l-glutamine, 1% sodium pyruvate, and 1% 2-mercaptoethanol [all Gibco, Grand Island, NY]) and expanded in vitro until passage 3. For injections, MSCs were lifted, counted, and resuspended (1×10^6 and 5×10^6 cells) in 1 mL of phosphate-buffered saline for injection.

AD-MSC were isolated from inguinal adipose tissue obtained from 6-week- to 8-week-old BN rats and digested enzymatically with collagenase type II (Worthington Biochemical Corp, Lakewood, NJ) and bovine serum albumin (Millipore, Billerica, MA) in Hanks balanced saline solution (Cellgro Mediatech Inc. Manassas, VA) for 60 minutes at 37°C. After centrifugation, the cellular pellet (stromal vascular fraction) was erythrocyte-depleted by lysis buffer, filtered, and transferred complete medium (Dulbecco's modified Eagle's medium/F-12, 10% fetal bovine serum, 1% penicillin/streptomycin, 1.25 mg/L amphotericin-B [all Gibco], 0.1 μM dexamethasone [Sigma-Aldrich, St. Louis, MO]). The AD-MSCs were allowed to attach for 6 hours, washed with phosphate-buffered saline, and expanded until the third passage.

Characterization of BM-MSC and AD-MSC

Expanded cells were stained for CD29, CD73, CD90, CD45, and RT1b (MHC I) and analyzed using a fluorescence-activated cell sorter (Aria; Becton Dickinson, Franklin Lakes, NJ). Data were analyzed using FlowJo software (TreeStar Inc., Ashland, OR).

MSC Treatment Protocol

Recipient animals were assigned to five groups: controls (n = 5), 1×10^6 BM-MSCs (n = 6), 5×10^6 BM-MSCs (n = 7), 1×10^6 AD-MSCs (n = 8), and 5×10^6 AD-MSCs (n = 9). All recipients were treated with rabbit anti-rat lymphocyte serum (Cedarlane, Burlington, NC) 4 days before and 1 day after surgery. Daily immunosuppression with tacrolimus (Astellas, Chicago, IL) (FK-506; 0.5 mg/kg) was administered intraperitoneally from day 0 to postoperative day 21. The treatment groups received a single MSC injection intravenously (i.v.)

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on postoperative day 1, injected slowly through the penile vein using a 30-gauge needle. Animals were observed daily for signs of rejection, assessed according to clinical VCA rejection grading which is based on graft gross appearance.[16] When rejection grade III was reached (experimental endpoint), animals were killed for tissue sampling. In two nonrejecting animals, BN donor skin grafts were transplanted to the neck to confirm donor-specific tolerance after 120 days.

Analysis of Inflammatory Mediators

Rat inflammatory mediators were measured using a Luminex 100 IS apparatus (Luminex, Austin, TX) and the rat multiplex Luminex assay (Affymetricvs/Panomics, Santa Clara, CA) as per manufacturer's specifications. The antibody bead kit included: β nerve growth factor (β NGF), Eotaxin (CCL11), granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), keratinocyte-derived cytokine (Gro-α/KC/CXCL1), intercellular adhesion molecule-1 (ICAM-1), interferon-γ (IFN-γ), interleukin (IL)-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IL-17A, IFN-γ–inducible protein 10 (IP-10/CXCL10), leptin, monocyte chemoattractant protein (MCP) (MCP-1/CCL2, and MCP-3/CCL7), macrophage inflammatory protein-1α (MIP-1α/CCL3), MIP-2/Gro-β, tumor necrosis factor-α (TNF-α), regulated on activation, normal T cell expressed and secreted (RANTES/CCL5), receptor activator of nuclear factor κ-B Ligand (RANKL), vascular cell adhesion molecule 1 (VCAM-1) and vascular endothelial growth factor-A (VEGF-A). The final mediator concentrations are expressed in pg/mL. Experimental data are presented as mean ± SEM.

Statistical and Computational Analyses

(1) Two-way analysis of variance (ANOVA) was carried out to analyze the changes in inflammatory mediators using SigmaPlot (Systat Software, San Jose, CA) as indicated.

(2) Principal component analysis (PCA) and time-interval PCA (TI-PCA) were carried out to identify the inflammatory mediators that contributed the most to the overall variance of the response in serum of rats that underwent hind-limb VCA along with i.v. injection of AD-MSCs or BM-MSCs (4, 6 and 18 weeks) using normalized data, as described.[17] Data were not binned. PCA and TI-PCA were performed using Matlab software (The MathWorks, Inc., Natick, MA).[17]

(3) Dynamic Network Analysis (DyNA) was carried out to define the central inflammatory network nodes as a function of both time and treatment. Rats underwent hind-limb VCA along with i.v. injection of AD-MSCs or BM-MSCs as described and serum samples were obtained at 4, 6, 18 weeks. Using inflammatory mediator measurements of three timepoints for each experimental group, networks were created over two consecutive periods (4–6 weeks and 6–18 weeks) using Matlab software;[17,18] data were not binned. Connections (or number of trajectories of serum inflammatory mediators that move in parallel) were created if the Pearson correlation coefficient between any two nodes (inflammatory mediators) at the same time interval was greater or equal to a threshold of 0.7 or 0.95, as indicated. The network complexity for each time interval was calculated using the following formula: Sum $(N_1 + N_2 + \ldots + N_n)/n-1$, where N represents the number of connections for each mediator, and n is the total number of mediators analyzed.

(4) Dynamic Bayesian Network (DyBN) inference was carried out to define the most likely single network structure that best characterizes the dynamic interactions among systemic inflammatory mediators across all timepoints, in the process suggesting likely feedback structures that define central nodes. The networks may also suggest possible mechanisms by which the progression of the inflammatory response differs within a given experimental subgroup. In this analysis, time courses of unprocessed inflammatory mediator measurements were used as input for a DyBN inference algorithm, implemented in Matlab essentially as described previously for gene array data[19] and modified by our group for the study of systemic acute inflammation.[20–22]

RESULTS

We have described the differential impact of AD-MSC and BM-MSC on VCA.[3,4] Herein, we sought to define the impact of these MSC populations on dynamic networks of systemic inflammation and immunity.

Characterization of AD-MSC and BM-MSC

The MSCs from BM and adipose tissue were characterized using flow cytometry. Both cell lines were negative for the hematopoietic marker CD45 (0.46% for BM-MSCs and 0.48% for AD-MSCs. Both cell types expressed high levels of CD29 (99.7% vs. 97.1%), CD90 (98.0% vs. 94.3%), and CD73 (93.6% vs. 90.5%).

Effects of AD-MSCs Versus BM-MSCs on Circulating Levels of Inflammatory Mediators After VCA

We hypothesized that the effects of MSCs on suppressor function, Treg cell induction, and chimerism we observed were associated with an altered balance of systemic inflammatory mediators after VCA. Accordingly, we initially analyzed the dynamics of circulating cytokines/chemokines by two-way ANOVA. The time-courses of inflammatory mediators are shown in Suppl. Fig. 1 (see Figure., Supplemental Digital Content 1, http://links.lww.com/TA/A939). This analysis suggested different dynamics of multiple mediators, but did not reveal statistically significant differences between the two experimental groups (all P values > 0.05).

DyNA of Inflammatory Mediators Suggests a Differential Network Complexity in AD-MSCs Versus BM-MSCs After VCA

Since standard statistical analysis of the inflammatory mediator data did not serve to differentiate between AD-MSCs and BM-MSCs after VCA, and given that the data are diverse, complex, and, for many mediators, nonlinear, we applied more advanced computational tools aimed at discerning principal characteristics and dynamic networks.[17,18,20]

DyNA was used to discern novel and potentially non-intuitive central inflammatory mediators involved in, or biomarkers of, the response to VCA across the three time points after treatment

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with either AD-MSCs or BM-MSCs. As shown in Figure 1, dynamic network complexity decreased with time in rats treated with AD-MSCs. These networks involved a large number of mediators (in decreasing order of connectivity) as depicted in Table 1. In contrast, DyNA network complexity increased as a function of time in rats treated with BM-MSCs (Fig. 2), and involved a large number of mediators (in decreasing order of connectivity) as depicted in Table 2.

DyBN Inference Suggests Different Motifs in AD-MSCs Versus BM-MSCs After VCA

We next used DyBN inference[18,20,22,23] to describe the overall feedback structure of the systemic inflammatory networks associated with AD-MSC versus BM-MSC and to discern potential central inflammatory nodes in response to AD-MSCs versus BM-MSCs. In rats subjected to VCA + AD-MSCs, central nodes (those network mediators exhibiting self-feedback as well as multiple effects on downstream nodes) were inferred to be ICAM-1, Leptin, and MIP-2 (Fig. 3*A*). In contrast, in rats subjected to VCA and BM-MSCs, central nodes were inferred to be VCAM-1, Leptin, MIP-2, and RANKL (Fig. 3*B*). These findings partially confirmed the findings obtained by DyNA (Tables 1 and 2).

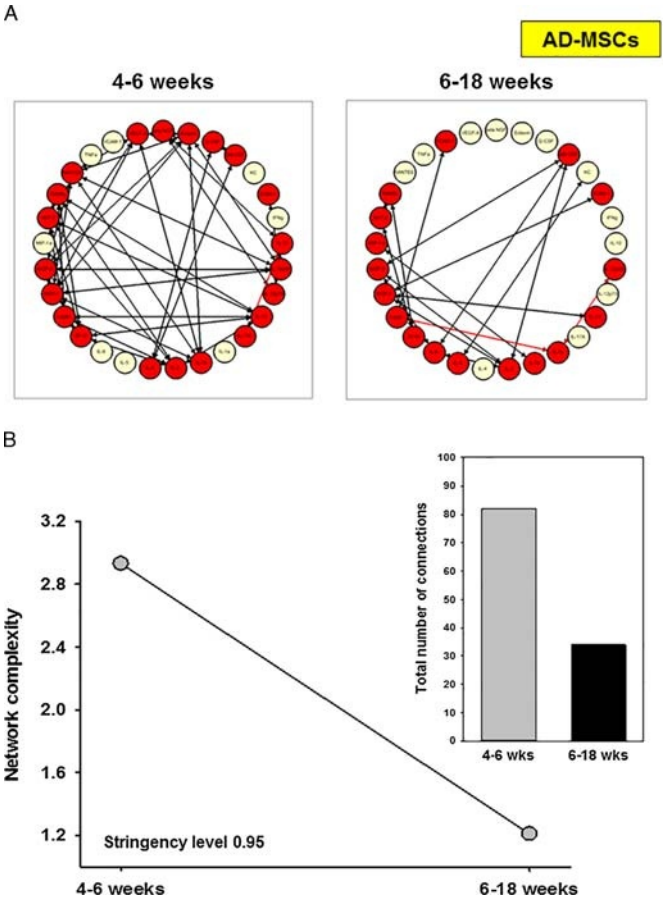

Figure 1. DyNA network complexity in rats undergoing VCA along with AD-MSCs. Rats underwent hindlimb VCA along with i.v. injection of 5×10^6 PKH26 (red) dye-labeled BN rat AD-MSCs as described in Materials and Methods. Serum samples were collected at 4, 6, 18 weeks and assayed for 29 inflammatory/immune mediators using the rat multiplex Luminex assay. DyNA was performed during the following two timeframes: 4 weeks to 6 weeks and 6 weeks to 18 weeks as indicated. (*A*) An overview of the networks and mediator connections (stringency level = 0.95). (*B*) The network complexity calculated as described in Materials and Methods.

TABLE 1. Rats Underwent Hindlimb VCA Along With i.v. Injection of 5×10^6 PKH26 (*red*) Dye-Labeled BN Rat AD-MSCs as Described in *Materials and Methods*

Mediators	4–6 weeks	6–18 weeks	Total
IL-2	5	4	9
MCP-1	6	3	9
MCP-3	5	3	8
IP-10	5	2	7
Leptin	5	2	7
MIP-2/GROβ	5	2	7
RANKL	5	2	7
Eotaxin/CCL11	6	0	6
IL-12/IL-23p40	5	1	6
IL-13	5	1	6
RANTES	6	0	6
GM-CSF	2	3	5
IL-1β	4	1	5
VEGF-A	5	0	5
IL-10	3	0	3
IL-6	0	3	3
β NGF	2	0	2
G-CSF	2	0	2
IL-12p70	2	0	2
ICAM-1	1	1	2
IL-1α	0	2	2
IL-4	2	0	2
GROα/KC	0	1	1
IL-17A	1	0	1
IL-5	0	1	1
MIP-1a	0	1	1
VCAM-1	0	1	1
IFN-γ	0	0	0
TNF-α	0	0	0

Serum samples were collected at 4 weeks, 6 weeks, 18 weeks, and assayed for 29 inflammatory/immune mediators using the rat multiplex Luminex assay. DyNA was performed during the following two timeframes: 4 weeks to 6 weeks and 6 weeks to 18 weeks as indicated. Table shows the number mediator connections (stringency level = 0.95) as determined by DyNA.

Differential Principal Characteristics of Inflammation in AD-MSCs Versus BM-MSCs After VCA Based on PCA and Time Interval PCA

Finally, we used PCA to define the primary dynamic characteristics of systemic inflammation in response to AD-MSCs versus BM-MSCs across these same time points (Fig. 4). This analysis suggested that the primary inflammatory characteristics or mechanisms of VCA + AD-MSCs involved IL-1β, RANTES, IL-2, IL-6, and eotaxin (Fig. 4*A*). In contrast, the primary

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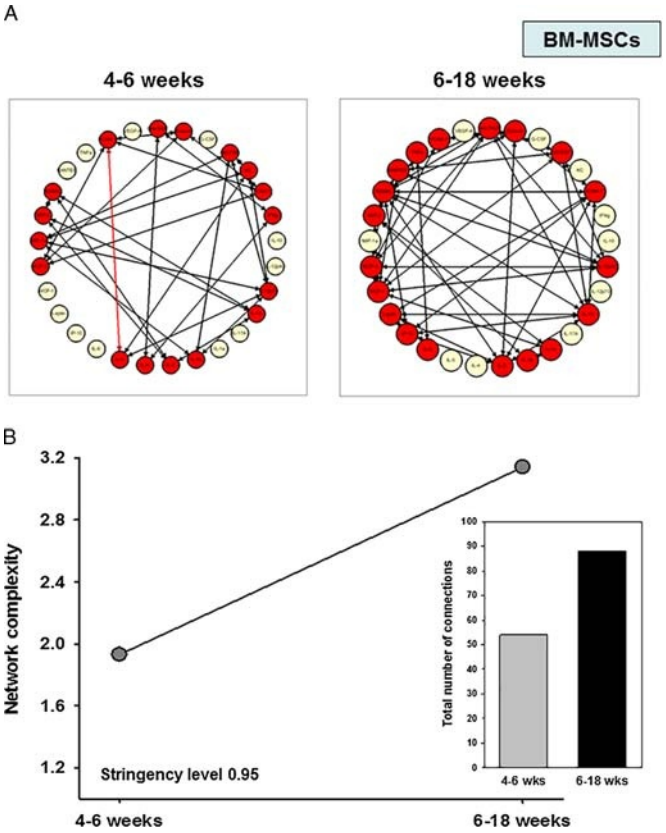

Figure 2. DyNA network complexity in rats undergoing VCA along with BM-MSCs. Rats underwent hind-limb VCA along with i.v. injection of 5×10^6 PKH26 (red) dye-labeled BN rat BM-MSCs as described in Materials and Methods. Serum samples were collected at 4 weeks, 6 weeks, 18 weeks, and assayed for 29 inflammatory/immune mediators using the rat multiplex Luminex assay. DyNA was performed during the following two timeframes: 4 weeks to 6 weeks and 6 weeks to 18 weeks as indicated. (*A*) An overview of the networks and mediator connections (stringency level = 0.95). (*B*) The network complexity calculated as described in Materials and Methods.

inflammatory characteristics or mechanisms of VCA/BM-MSCs were inferred to involve IL-13, RANKL, MIP-2, RANTES, and IL-2 (Fig. 4*B*). Furthermore, we developed a method (time interval PCA; TI-PCA) to identify those inflammatory mediators that contributed the most to the overall variance of the inflammatory response in both experimental groups over two consecutive periods (4–6 weeks and 6–18 weeks). The results of this analysis and the top five inflammatory mediators for each time interval are shown in Figure 4*C* (AD-MSCs) and Figure 4*D* (BM-MSCs).

DISCUSSION

The present study was aimed at defining, at least in part, some of the modulatory effects of AD-MSC and BM-MSC on VCA-induced systemic inflammation. Our results suggest that, in concert with prolonging survival of immunogenic skin-bearing VCA grafts,[4] these different MSC types exert variable immunosuppressive effects along with distinct impacts on dynamic inflammatory network complexity.

In vivo, all animals revealed peripheral multilineage chimerism at 4 weeks ($p < 0.01$) independent of cell type and dosage.[4] These initial studies also showed that both AD-MSC and BM-MSC could drive a strong, dose-dependent suppressor function in vitro, though this effect was significantly more pronounced for AD-MSC.[4] This, in concert with more pronounced Treg cell levels and better survival in AD-MSC as compared to BM-MSC, led us to hypothesize that AD-MSC somehow impacted VCA-induced systemic inflammation differentially.

Our findings based on DyNA suggest increasing dynamic evolution of networks of systemic inflammation in rats undergoing VCA. We found that dynamic network complexity decreased with time in rats treated with AD-MSC, whereas the opposite was true in rats treated with BM-MSC. The significance of this finding is currently under investigation. However, we have suggested previously that trends toward increasing inflammatory network complexity as assessed by DyNA is often associated with adverse outcomes such as mortality after

TABLE 2. Rats Underwent Hindlimb VCA Along With i.v. Injection of 5×10^6 PKH26 (*red*) Dye-Labeled BN Rat BM-MSCs as Described in *Materials and Methods*

Mediators	4–6 weeks	6–18 weeks	Total
RANKL	3	8	11
IL-13	3	7	10
β NGF	2	7	9
GM-CSF	4	5	9
ICAM-1	3	6	9
IL-2	3	6	9
MCP-3	3	5	8
Eotaxin/CCL11	3	4	7
MCP-1	0	7	7
MIP-2/GROβ	3	4	7
IL-12/IL-23p40	0	6	6
VCAM-1	4	2	6
IL-12p70	5	0	5
IL-1β	3	2	5
IP-10	0	5	5
RANTES	0	5	5
GROα/KC	4	0	4
Leptin	0	4	4
MIP-1a	4	0	4
IL-1α	0	3	3
IL-5	3	0	3
IFN-γ	2	0	2
IL-4	2	0	2
IL-6	0	1	1
TNF-α	0	1	1
G-CSF	0	0	0
IL-10	0	0	0
IL-17A	0	0	0
VEGF-A	0	0	0

Serum samples were collected at 4 weeks, 6 weeks, 18 weeks, and assayed for 29 inflammatory/immune mediators using the rat multiplex Luminex assay. DyNA was performed during the following two time frames: 4 weeks to 6 weeks and 6 weeks to 18 weeks as indicated. Table shows the number mediator connections (stringency level = 0.95) as determined by DyNA.

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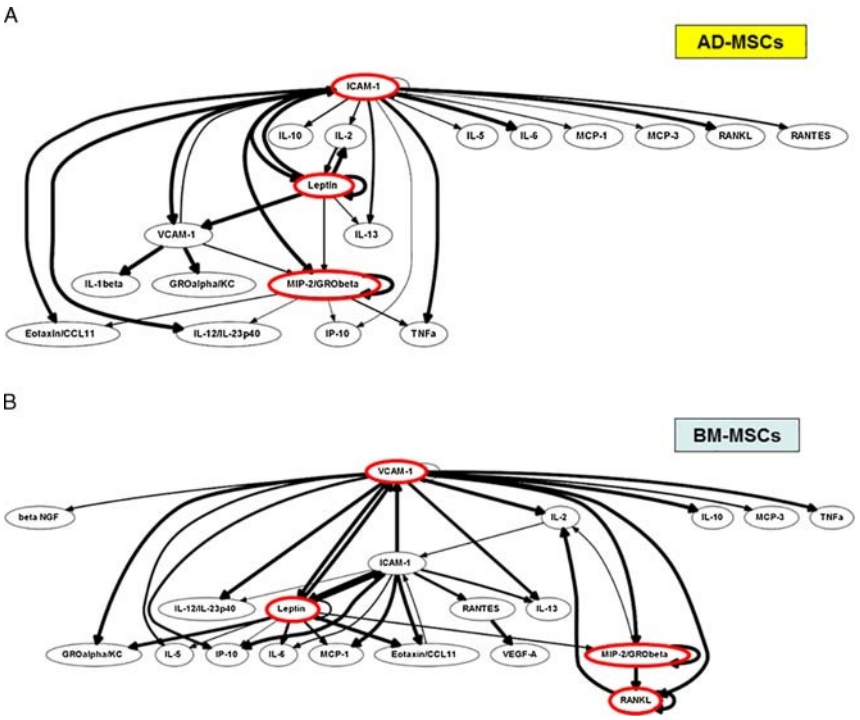

Figure 3. Differential DyBN network patterns in rats undergoing VCA along with AD-MSCs vs. BM-MSCs. Rats underwent hind-limb VCA along with i.v. injection of 5×10^6 PKH26 (red) dye-labeled BN rat AD-MSCs or BM-MSCs as described in Materials and Methods. Serum samples were obtained at 4 weeks, 6 weeks, 18 weeks, and assayed for 29 inflammatory/immune mediators using the rat multiplex Luminex assay followed by DyBN analysis as described in Materials and Methods. (*A* and *B*) The inflammatory networks for AD-MSCs and BM-MSCs, respectively.

trauma[24] or in the setting of pediatric acute liver failure.[25] In the trauma setting, increasing inflammatory network complexity was paralleled by increasing multiple organ dysfunction.[24] We thus hypothesize a potentially greater benefit of AD-MSC vs. BM-MSC. In support of this hypothesis, AD-MSC treatment resulted in long-term (>120 day) allograft survival in 47% of the animals, which correlated with durable microchimerism in bone marrow and spleen.[4] Although both AD-MSC and BM-MSC exhibited strong, dose-dependent suppressor function in vitro, this was significantly more pronounced for AD-MSC.[4] Finally, Treg cell levels were increased with both cell types, but to a higher degree in AD-MSC rats,[4] again suggesting a potentially greater benefit for AD-MSC in VCA (summarized in Fig. 5; see further discussion below). However, long-term immunomodulatory effects remain uncertain and require further study.

In parallel, we have found that dynamic inflammatory networks start out at higher complexity and then decrease in trauma patients who resist nosocomial infections.[26] This and other prior studies have raised the possibility of positive feedback in the systemic inflammatory response that is sustained or promoted by multiple pathways, including innate immune and Th17-related mechanisms, and kept in check via Treg cells.[24] These hypotheses are supported by our current data in VCA: rats treated with AD-MSC exhibit decreasing complexity of systemic inflammatory networks in concert with elevated Treg cells, whereas animals treated with BM-MSC display the opposite phenotype. Further studies are needed, however, to help prove or disprove this hypothesis.

Our analyses as a whole suggested that multiple cytokines and chemokines were differentially interconnected in rats treated with AD-MSC versus BM-MSC. Based on DyNA, the top 10 most connected mediators in rats treated with AD-MSC included IL-2, MCP-1, MCP-3, IP-10, leptin, MIP-2, eotaxin, IL-12, IL-13, and RANTES. In rats treated with BM-MSC, the top 10 most connected mediators were RANKL, IL-13, β-NGF, GM-CSF, ICAM-1, IL-2, MCP-3, eotaxin, MCP-1, and MIP-2. These differential connectivity patterns suggest a conserved signature of response to VCA with or without treatment with MSCs, consisting of pathways driven by IL-2, IL-13, MCP-1, MCP-3, MIP-2, and eotaxin. AD-MSC–specific systemic inflammation appears to include pathways driven by IP-10, leptin, IL-12, and RANTES, whereas BM-MSC appear to stimulate pathways driven by RANKL, β-NGF, GM-CSF, and ICAM-1.

Our data show that IL-2 is an important driver of networks after AD-MSC treatment. Activated T cells produce IL-2. Treg cells abundantly express the IL-2 receptor, and IL-2 is a driver of Treg cell suppressor activity. This suggests that perhaps this mediator is a hallmark of Treg cell induction by MSCs. Our prior findings in VCA confirmed that both AD-MSC and BM-MSC exhibited strong, dose-dependent suppressor function in vitro, which was significantly more pronounced for AD-MSC. In vivo, all animals revealed peripheral multilineage chimerism at 4 weeks, independent of cell type and dosage. Treg cell levels were increased with both cell types, but more in the AD-MSC treatment groups.[4] Furthermore, AD-MSC treatment resulted in

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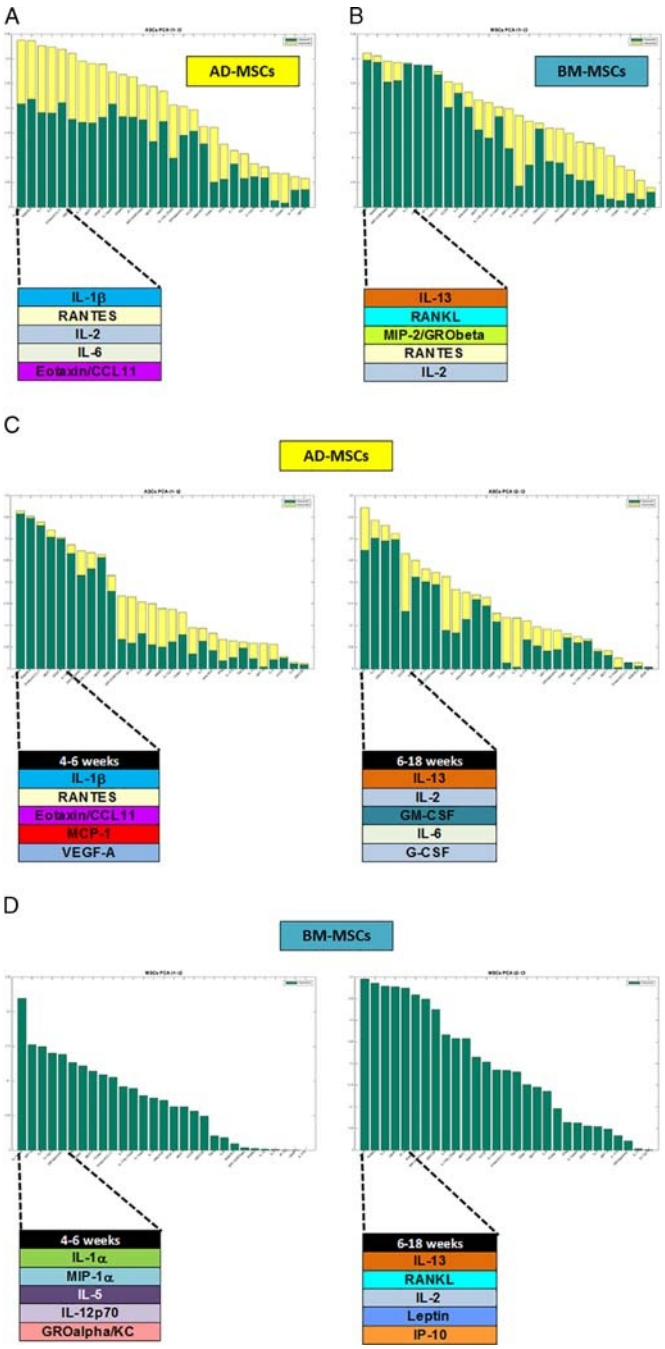

Figure 4. Differential primary inflammatory characteristics in rats undergoing VCA along with AD-MSCs versus BM-MSCs as discerned by PCA and TI-PCA. Rats underwent hindlimb VCA along with i.v. injection of 5×10^6 PKH26 (red) dye-labeled BN rat AD-MSCs or BM-MSCs as described in Materials and Methods. Serum samples were obtained at 4 weeks, 6 weeks, 18 weeks, and assayed for 29 inflammatory/immune mediators using the rat multiplex Luminex assay followed by PCA and TI-PCA as described in Materials and Methods. (*A* and *B*) The inflammatory characteristics of AD-MSCs and BM-MSCs, respectively. The top five inflammatory mediators are highlighted for both groups. (*C* and *D*) The inflammatory characteristics and top five inflammatory mediators for each time interval of AD-MSCs and BM-MSCs, respectively.

long-term (>120 day) allograft survival in 47% of the animals, which correlated with durable microchimerism in bone marrow and spleen.[4]

Both AD-MSC and BM-MSC are known to induce Treg cells from effector T cells as well as expanded natural Treg (nTreg) cell. nTreg and induced Treg (iTreg) cells can be distinguished by not only their origin but also by differences in the expression of the transcription factor, FOXP3 and methylation pattern of the Treg cell–specific demethylated region in the FOXP3 gene.[27] Both Treg cell types have similar immunoregulatory effects, but a longer lifespan, when compared to MSC, which is only approximately 24 hours after infusion.[28] The superior efficacy of AD-MSC versus BM-MSC in prolonging graft survival in our study possibly relates to their differential ability to sustain the persistence of Treg cells in the recipients long after administration, as well as the dynamic inflammation networks that emerge thereafter in the presence of increased numbers of Treg cells. Although we did not study the expression or methylation patterns of FOXP3 in Treg cell subsets in our study, we postulate that AD-MSC-stimulated iTreg cells might help drive an anti-inflammatory program that is in part driven by IP-10 (as we have suggested previously, based on analysis of dynamic networks, in the response to traumatic injury). In contrast, we hypothesize that nTreg cells are predominantly induced by BM-MSC and are consequently less effective at reducing rejection (Fig. 5). This hypothesis will be a focus of future studies in our lab.

It is also tempting to speculate that BM-MSC may help drive nerve regeneration based on the triggering of β-NGF. RANTES is a chemokine that is involved in multifaceted chemoattraction of lymphocytes, and has been associated with chronic rejection in the context of transplantation.[29] Interestingly, this chemokine has also been reported to be elevated after treatment with a human BM-MSC line in a spinal cord injury paradigm,[30] supporting the emerging concept that the immunomodulatory properties of MSCs may be context-specific.[31]

It is important to note that the foregoing hypotheses, and potentially numerous others, could not have been derived without the use of advanced computational modeling techniques as used in the current study. Static cytokine measurements of inflammation or rejection remain the conventional accepted standard in research. However, such methods failed to show statistically significant differences in the current study because they are not capable of discerning the complex dynamic interconnectivity among the myriad mediators as analyzed. Further studies with a larger panel of mediators are needed to test these hypotheses and better define the potential mechanisms that underlie the in vivo efficacy of AD-MSC versus BM-MSC therapies in the setting of VCA. However, our results demonstrate the utility of our computational methods for suggesting novel hypotheses that can be tested in future studies.

Strategies aiming at minimization or elimination of systemic immunosuppression are key immediate goals for clinical expansion of VCA. The importance and relevance of dynamic network complexity during inflammation, and their relation to outcomes in related fields, such as traumatic injury,[22,24,26,32–34] reinforces the rationale for similar studies in VCA. Our in silico results offer key primary insights into similar mechanisms in VCA. Further exploration of key drivers as well as complexity

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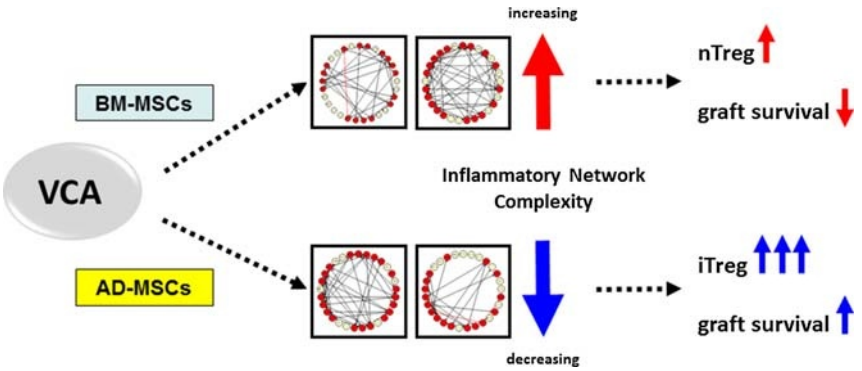

Figure 5. Hypothetical overview of the concordance among dynamic inflammatory network complexity, activation of Treg cells, and ultimate graft survival outcomes after VCA. The survival differences between AD-MSC and BM-MSC may relate to differences in augmentation of iTreg cells as compared to nTreg cells.

of cytokine networks during early inflammatory or immune events after VCA (such as ischemia reperfusion injury and surgical inflammation) may help identify promising cellular or molecular targets for rational treatment interventions in VCA.

AUTHORSHIP

R.Z. and S.K.R. contributed equally to this work. Y.V. and V.S.G. are senior co-authors. R.Z. participated in computational and statistical analysis, data interpretation, writing. S.K.R. participated in in vitro experiments, data collection, and analysis. J.A.P. participated in in vivo experiments and data collection. Y.V. participated in study design, literature search, data interpretation, and writing. V.S.G. participated in study design, literature search, data interpretation, and writing.

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DISCLOSURE

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